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Nuclear Signaling by Receptor Tyrosine Kinases: The First Robin of Spring

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The role of receptor tyrosine kinases (RTKs) in transmembrane signaling is well established. Recently, ligand-dependent translocation of RTKs to the nucleus has been reported, but the functional importance of this process remains unclear. In this issue, Sardi et al. (2006) provide evidence for direct signaling in the nucleus by an intracellular ErbB4 receptor fragment that is released upon receptor activation by ligand. The fragment forms a complex with the adaptor TAB2 and the corepressor N-CoR and transits to the nucleus, where it represses transcription of genes that promote the formation of astrocytes.

Following discovery of the first growth factors, nerve growth factor (NGF) and epidermal growth factor (EGF), it became clear that NGF, EGF, and hormones such as insulin mediate their distinct pleiotropic responses by binding to and activating cell-surface receptors endowed with intrinsic tyrosine kinase activity, designated receptor tyrosine kinases (RTKs) (reviewed in Schlessinger, 2000; Blume-Jensen and Hunter, 2001). RTKs transmit the activation signal across the plasma membrane, and many studies have demonstrated that the receptors, and not the growth factors, mediate the pleiotropic cellular responses. Growth factors recognize and activate their cognate receptors and stimulate receptor dimerization, tyrosine kinase activation, and autophosphorylation (reviewed in Schlessinger, 2000). The autophosphorylated RTKs recruit and activate a receptor-specific complement of intracellular signaling pathways that relay information to the nucleus and other intracellular compartments (Figure 1) (reviewed in Schlessinger, 2000; Blume-Jensen and Hunter, 2001; Pawson et al., 2001; Levy and Darnell, 2002).

Beyond this well-established mechanism of RTK signaling, direct communication between the membrane and nucleus by ligand-dependent nuclear localization of RTKs has also been reported. In a few cases, such as the EGF receptor, nuclear translocation of the intact receptor has been proposed, although the mechanistic basis for this, and its functional relevance, is extremely unclear (reviewed in Carpenter, 2003a; Krolewski, 2005; Massie and Mills, 2006). By contrast, Carpenter's group has shown that ErbB4, an RTK that binds to neuregulin 1, is cleaved by a dual-protease system following the binding of ligand. Ligand binding promotes an initial cleavage event in the ErbB4 extracellular juxtamembrane domain (mediated by tumor necrosis factor α-converting enzyme, or TACE), followed by a second cleavage event within the transmembrane domain that is mediated by presenilin/γ-secretase (Figure 1). An active soluble form of the ErbB4 cytoplasmic region is thus liberated (Carpenter, 2003a, 2003b) and is translocated into the nucleus with potential functional consequences (Ni et al., 2001). A similar mechanism involving sequential cleavages in the juxtamembrane and transmembrane domains was previously established for the membrane-bound proteins Notch, amyloid precursor protein (APP), and sterol regulatory element-binding protein 2 (SREBP2). SREBP2 is a membrane-bound cholesterol sensor that acts as a transcription factor in the nucleus (Goldstein et al., 2006). In dual-protease processing of SREBP2, the first cleavage is performed by a serine protease belonging to the subtilisin family, and the second is mediated by a Zn^{2+} metalloproteinase. It was shown that the liberated soluble form of SREBP2 contains a nuclear localization signal (NLS), a DNA binding domain, and a transcriptional activation domain that activates genes required for cholesterol production (Goldstein et al., 2006).

In this issue of *Cell*, Sardi et al. (2006) describe a mechanism initiated by dual-protease signaling for the regulation of gene transcription and cell fate through direct nuclear signaling by an ErbB4 fragment. Transcriptional repression by a complex involving this ErbB4 fragment controls the timing of astrogenesis in the developing cerebral cortex of mice.

Canonical ErbB4 Signaling at the Plasma Membrane

ErbB4 is a member of the EGF receptor (EGFR) family of RTKs (Plowman et al., 1993), which are activated at the cell membrane by neuregulins and other growth factors such as betacellulin (BTC) and heparin-binding EGF-like growth factor (reviewed in Carpenter, 2003b). Like other RTKs, ErbB4 is composed of an extracellular ligand-binding region that is connected to a cytoplasmic portion via a single transmembrane (TM) domain (reviewed in Schlessinger, 2000; Carpenter, 2003b). The cytoplasmic portion of ErbB4 contains a conserved tyrosine kinase domain

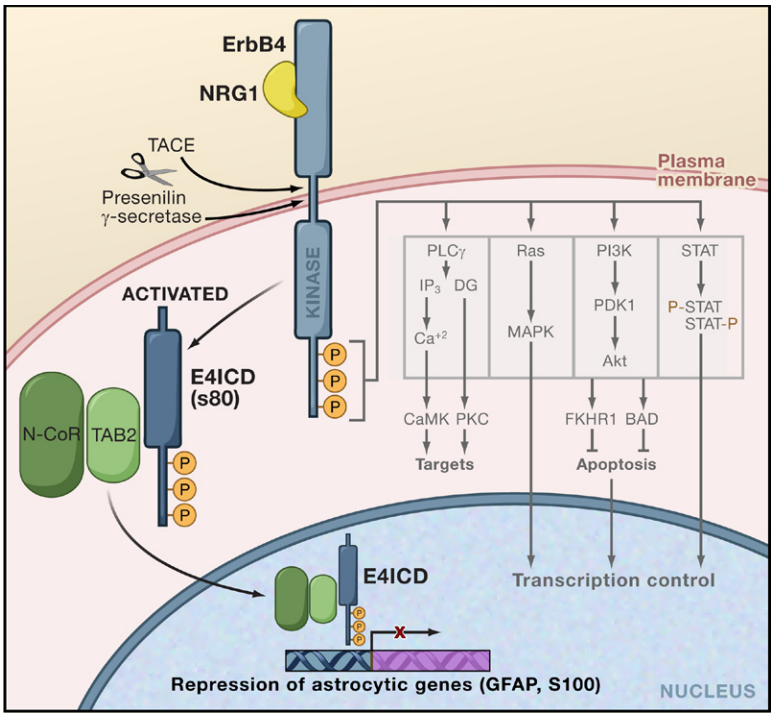

Figure 1. Canonical and Nuclear ErbB4 Signaling Pathways

In the canonical signaling pathways activated by ErbB4 and other receptor tyrosine kinases, signal transduction is initiated by the intact receptor. New work by Sardi et al. (2006) shows that, in addition to its canonical functions, a cleaved intracellular domain of the JM-a isoform of ErbB4 translocates to the nucleus and represses gene transcription. Binding of neuregulin 1 (NRG1) to the extracellular ligand-binding region of ErbB4 stimulates receptor dimerization, tyrosine kinase activation, autophosphorylation, and recruitment of signaling proteins such as the adaptor proteins Shc and Grb2. Receptor stimulation leads to the activation of the Ras/MAPK, PI3K/Akt, and phospholipase Cγ (PLCγ) cascades and the phosphorylation of STAT proteins. The PI3K/Akt signaling cascade plays a critical role in stimulation of an antiapoptotic response. Upon tyrosine phosphorylation and activation, PLCγ stimulates phosphatidylinositol-(4,5)-bisphosphate hydrolysis and Ca^{2+} release from cytoplasmic stores, leading to activation of serine/threonine kinases. STAT proteins dimerize upon tyrosine phosphorylation and move to the nucleus to regulate gene transcription. When stimulated by neuregulin 1, the activated JM-a isoform of ErbB4 is also cleaved by TACE in the extracellular juxtamembrane region, releasing the ligand-binding domain of the receptor. The truncated receptor at the cell membrane is then cleaved in its transmembrane domain by a presenilin γ-secretase to liberate an activated form of the ErbB4 intracellular domain, designated s80 or E4ICD. E4ICD interacts with the adaptor protein TAB2 that is bound to nuclear receptor corepressor (N-CoR). The ternary E4ICD/TAB2/N-CoR complex then moves to the nucleus, where it represses genes such as *GFAP* and *S100* that prevent neuronal progenitor cells from developing into astrocytes.

flanked by regulatory sequences that become tyrosine autophosphorylated in response to ligand stimulation and are also phosphorylated by several serine/threonine protein kinases. Autophosphorylated tyrosines in the C-terminal regulatory region of ErbB4 act as docking sites for the adaptor proteins Grb2 and Shc and signaling proteins such as phospholipase Cγ (PLCγ), phosphatidylinositol 3-kinase (PI3K), and signal transduction and activators of transcription (STATs). The recruitment of these proteins activates downstream signaling pathways (Figure 1) (reviewed in Schlessinger, 2000; Blume-Jensen and Hunter, 2001; Pawson et al., 2001; Levy and Darnell, 2002) that mediate many cellular processes.

Nuclear Translocation of a Cleaved ErbB4 Intracellular Domain

A unique feature of ErbB4 that sets this receptor apart from other EGFR family members is the existence of two alternatively spliced isoforms with different sequences in the extracellular juxtamembrane region, designated JM-a and JM-b (Elenius et al., 1997). The JM-a and JM-b isoforms of ErbB4 exhibit different tissue expression patterns. The JM-a isoform is expressed exclusively in the kidney, uterus, and eye, whereas the JM-b isoform is expressed exclusively in heart and cerebral cortex; both isoforms are expressed in the cerebellum and spinal cord (Elenius et al., 1997). Whereas the JM-b isoform of ErbB4 functions primarily as a canonical RTK and signals from the cell surface, the JM-a isoform additionally employs the dual-protease (TACE/presenilin) signaling mechanism as described above, making it unique among RTKs. Stimulation with neuregulin at the cell surface promotes extracellular cleavage of the JM-a isoform of ErbB4 by TACE (Rio et al., 2000), which cleaves ErbB4 between His651 and Ser652 and liberates a soluble form of the extracellular ligand-binding region (reviewed in Carpenter, 2003b). The remaining membrane-anchored portion of ErbB4 (m80) is further cleaved within its transmembrane domain by a presenilin γ-secretase (Figure 1) to produce a soluble dimeric, active form of the intracellular domain (ICD) of ErbB4, designated E4ICD or s80 (Ni et al., 2001; Lee et al., 2002; Carpenter, 2003b; Linggi and Carpenter, 2006; Linggi et al., 2006). Analysis of the cellular localization of E4ICD using fluorescence microscopy has demonstrated that E4ICD is translocated into the nucleus.

Nuclear ErbB4 Represses Transcription of Neuronal Differentiation Genes

Sardi et al. (2006) used an activated form of E4ICD as a yeast two-hybrid bait for screening a cDNA expression library derived from rat embryonic spinal cord and dorsal root ganglia. One of the proteins found to interact with E4ICD was TAB2, previously identified as an adaptor molecule that participates in signaling downstream of interleukin-1 (IL-1). TAB2 forms a complex with TAK1, a protein kinase that plays a key role in IL-1 stimulation of NF-κB and JNK activation (Takaesu et al., 2000). Importantly, Sardi et al. demonstrated that complex formation between TAB2 and E4ICD is specific and is dependent

on the tyrosine kinase activity of the ErbB4 fragment, although TAB2 does not appear to become phosphorylated in response to ErbB4 activation by neuregulin 1. TAB2 interacts with either the JM-a or JM-b isoform of (intact) ErbB4 in a neuregulin-dependent manner. However, translocation of TAB2 to the nucleus (as part of a TAB2/E4ICD complex) only occurs following presenilin-dependent cleavage of the JM-a isoform of ErbB4.

Consistent with previous studies demonstrating that TAB2 can also form a complex with the nuclear receptor corepressor N-CoR (Baek et al., 2002), a ternary E4ICD/TAB2/N-CoR complex could be detected in lysates from neuregulin 1-stimulated cells that express the JM-a isoform of ErbB4. TAB2 may act as a "bridge" or adaptor molecule, constitutively associating with N-CoR through its amino terminus and recruiting the TAB2/N-CoR complex to neuregulin 1-activated ErbB4 through its carboxyl terminus. Following presenilin-dependent cleavage of ErbB4 (JM-a), the TAB2/N-CoR complex remains associated with the liberated E4ICD, and the ternary E4ICD/TAB2/N-CoR complex undergoes nuclear translocation. Nuclear translocation is likely promoted by the nuclear localization sequence found in E4ICD because siRNA knockdown of TAB2 does not affect E4ICD nuclear localization. The nuclear translocation of the ternary E4ICD/TAB2/N-CoR complex represses transcription of several glial genes that are required for the differentiation of neuronal precursor cells into astrocytes, including *GFAP* and *S100*β, although the precise mechanism of this repression and the basis for promoter selectivity have yet to be determined. Neuregulin 1 stimulation of neuronal precursor cells antagonizes the ability of ciliary neurotrophic factor (CNTF) to promote astrogenesis, in part by repressing genes such as *GFAP* and *S100*β through the presenilin-dependent mechanism outlined above, thereby maintaining the neurogenic state. The canonical mode of ErbB4 signaling cannot elicit these effects.

Sardi et al. (2006) used RNAi knockdown of *TAB2* and *ErbB4* to demonstrate that these molecules are critical for the inhibitory effect of neuregulin 1 on astrogenesis and GFAP expression. Chromatin immunoprecipitation assays also demonstrated that E4ICD associates with the *GFAP* and *S100*β promoters in a neuregulin 1-dependent manner. From studies using genetically modified mice lacking ErbB4 in most tissues, the authors conclude that the timing of astrogenesis during embryonic development is determined by transcriptional repression promoted by direct nuclear action of the cleaved JM-a ErbB4 isoform. Astrogenesis occurs later in development than neurogenesis, and nuclear ErbB4 signaling appears to play a key role in creating this delay by inhibiting the onset of astrogenesis. Mice deficient in ErbB4 show precocious cortical astrogenesis, which can be rescued by expression of the cleavable JM-a isoform of ErbB4, but not by the JM-b isoform.

Intracellular signaling pathways that are activated by Notch, ErbB4, and other extracellular cues initiate a molecular program in the early stages of embryonic development that preferentially stimulates neurogenesis over astrogenesis. At this stage of embryonic development, astrogenesis is repressed by the presenilin-dependent action of the JM-a isoform of ErbB4. At a later stage of embryonic development, expression of the JM-a ErbB4 isoform in neuronal precursor cells is reduced, and this enables the induction of astrocyte development by other stimulatory signals (Fox and Kornblum, 2005). It is noteworthy that presenilin plays a positive role in the control of Notch-1 action by activating an intracellular signaling pathway that stimulates astrocyte development. It is expected, therefore, that a deficiency in presenilin will have only a minor effect on astrocyte development because the reduced stimulation of Notch signaling will be offset by the loss of inhibitory signaling through the JM-a isoform of ErbB4. Indeed, a minimal change in expression of markers for astrocyte development was detected in mice lacking presenilin.

Sardi et al. (2006) propose that the nuclear action of the JM-a isoform of ErbB4 also plays a role in the pathology of Alzheimer's disease (AD). This notion is based partly on the finding that ErbB4 is highly enriched in neuronal plaques of AD patients. Moreover, it is well established that APP, the major mediator of AD, is cleaved by a similar presenilin-dependent mechanism that results in the release of the APP cytoplasmic domain to mediate its intracellular effects. Interestingly, the presenilin-cleaved APP product interacts with TAB2 and N-CoR, the two partners of E4ICD shown to be responsible for mediating the transcriptional repression of astrocyte development. It is proposed that the common targets for the presenilin-cleaved forms of APP and ErbB4 may influence the transcriptional regulation of genes that lead to neurodegeneration.

Do Intact Receptors Move to and Act in the Nucleus?

There are also reports that intact RTKs may be translocated to the nucleus in a ligand-dependent manner (reviewed in Carpenter, 2003a; Krolewski, 2005; Massie and Mills, 2006), although our understanding of the mechanism underlying this process is extremely limited (Krolewski, 2005; Massie and Mills, 2006). In contrast to the convincing studies describing the signaling role of E4ICD in direct nuclear signaling by ErbB4, there has been no convincing demonstration that intact RTKs in the nucleus play an important signaling role.

It has been clear for decades that activated ligand/receptor complexes formed on the cell-surface cluster in coated pit invaginations that then pinch off to become coated vesicles, which carry the occupied activated receptors inside the cell via receptor-mediated endocytosis. However, is it possible that this provides a route for delivery of intact receptors to the nucleus? After endocytosis, most internalized ligand/RTK complexes are delivered to lysosomes for degradation or are recycled (following ligand dissociation) back to the cell surface for reutilization by a fresh pool of soluble ligand molecules. It now also seems clear that internalized RTKs can activate intracellular signaling pathways while residing in intracellular vesicles following endocytosis. However, it

is generally thought that the main function of internalization of ligand/RTK complexes is to terminate the signal that is initiated at the cell surface.

An alternative school of thought is that an important role of endocytosis is to deliver ligand/RTK complexes into the cell interior to enable their translocation into the nucleus, where intact growth factors, RTKs alone, or growth factor/RTK complexes participate directly in regulating transcriptional stimulation or repression of genes important in the control of cell proliferation, cell differentiation, and cell survival (reviewed in Krolewski, 2005; Massie and Mills, 2006). Indeed, a large body of literature has accumulated over the past 25 years on nuclear signaling by a variety of growth factors (e.g., NGF, EGF, and FGF), hormones (e.g., insulin), and RTKs (e.g., insulin receptor, EGF receptor, FGF receptor, and NGF receptor) as well as intact ligands bound to their cognate receptors. This large body of work has been received with some skepticism for two main reasons. First, in the context of our extensive knowledge of signaling by most of these receptors, it is currently not necessary to invoke direct nuclear action of the receptors (or ligands) to explain their biological activities, although this naturally does not prove a lack of nuclear function. Second, the mechanisms proposed for the various steps involved in the movement of growth factors and RTKs from endocytic vesicles to the cytoplasm and nucleus have not been convincing. For example, it was proposed that intact growth factors and RTKs that reside in the limiting membranes of endocytic vesicles might be extracted from these membranes into the cytoplasm through retrotranslocation. In this model, retrotranslocation is mediated by the Sec61 translocon (Carpenter, 2003a; Krolewski, 2005; Massie and Mills, 2006) in a process analogous to the endoplasmic reticulum (ER) associated degradation (ERAD) system that normally transports misfolded proteins from the ER to the cytoplasm for degradation by the ubiquitin proteasome system. How these receptors reach the ER (where Sec61 is located), or whether Sec61 is ever found in endocytic vesicles, is far from established. It has been pointed out (Carpenter, 2003a; Massie and Mills, 2006) that certain toxins and viral proteins are thought to gain access to the ER, cytoplasm, and/or nucleus through such mechanisms. However, it is not clear how large multidomain proteins such as EGFR and other RTKs can be moved across cell membranes via a channel that normally functions to transport unfolded or misfolded proteins across cell membranes. It is also not clear how RTKs that are transported by Sec61 into the cytoplasm would escape degradation by the Sec61-associated ubiquitination and proteasome system. Given these conceptual difficulties and the fact that the molecular targets of nuclear growth factors and receptors have not been adequately described, it may be that these reports reflect artifactual contaminants rather than genuine biologically significant components of the transcriptional apparatus.

The experiments presented in the report of Sardi et al. (2006) represent one of the first robins of spring in this field. In contrast with the intact receptor studies, Sardi et al. demonstrate clearly that ErbB4 participates in a biologically significant signaling mechanism mediated by direct nuclear action of an activated ErbB4 fragment that is transported from the cell membrane to the nucleus in a manner that is regulated by a known ErbB4 ligand. In addition to its biological significance and functional credibility, there is also now a sophisticated understanding of the molecular basis for this signaling process, although details remain to be elucidated. The collective work on ErbB4 nuclear signaling summarized here sets a standard with which to challenge all other studies of direct nuclear signaling by RTKs.

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